IN VITRO EVALUATION OF SELECTED INDIAN MEDICINAL PLANTS FOR ANTI DIABETIC PROPERTIES

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Summary

The present study is focused on evaluation of selected medicinal plants viz., *Eugenia jambolana, Centella asiatica, Ocimum sanctum* and *Coccinia indica* for potential inhibition of carbohydrate hydrolyzing enzymes such as α-amylase and α-glucosidase, and insulinotrophic properties in pancreatic β RIN m5F cells. Among the selected plants, *E. jambolana* and *C. indica* showed promising inhibitory activities against α-amylase and α-glucosidase enzymes tested in a dose dependent manner and insulinotrophic activity in pancreatic cell lines. The methanol leaf extract of *C. indica* exhibited highest α-amylase and α-glucosidase inhibitory activity with an IC_{50} value of 8.17 µg/mL and 16.75 µg/mL respectively. In *E. jambolana,* the methanol leaf extract showed α-glucosidase inhibitory activities with an IC_{50} of 16.27 µg/mL and α-amylase inhibitory activity with IC_{50} of 33.96 µg/mL. These extracts also showed insulinotrophic properties with more than 2-fold increase in insulin release, compared to glucose. The cytotoxic activity of the extracts on pancreatic β RIN m5F cells was carried out using MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) reagent. All the extracts were not showing any cytotoxicity effects even at higher concentrations. These results showed that these plants can be a promising source for development of carbohydrate blockers and novel insulin secretagogues.

Key words: Diabetes mellitus, α-amylase, α-glucosidase, medicinal plants,

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Introduction

Diabetes mellitus is an endocrine disorder in which inadequate release of insulin from pancreatic β-cells, or insensitivity of target tissue to insulin (NIDDM) and accounts for 90% of cases throughout the world [1,2]. The worldwide epidemic of type 2 diabetes has been stimulating the search for new concepts and targets for the treatment of this incurable disease. Several therapeutic strategies are adopted to decrease the blood glucose level. One promising therapeutic approach to decrease the hyperglycemia, is to retard and reduce the digestion and absorption of ingested carbohydrates through the inhibition of carbohydrate hydrolyzing enzymes (such as α-amylase, α-glucosidase). As a result, these inhibitors could decrease the postprandial rise in blood glucose concentration [3].

Synthetic drugs used for diabetes therapy are having side effects and do not restore normal glucose homeostasis [3]. Therefore, many efforts have been made to identify new antidiabetic agents from different sources, especially medicinal plants because of their effectiveness, fewer side effects and relatively low cost [4]. Thus, it is necessary to look for new and, more efficacious drugs and make use of the vast reserves of phytotherapy for medicinal purposes.

Ethnobotanical information indicated that about 800 plants have been reported to possibly possess anti-diabetic potential [2]. A number of plants are mentioned in ancient literature and some of them have been tested experimentally for antidiabetic properties [5, 6, 7, 8 & 9]. Thus plants serve to be a potential source of anti-diabetic drugs, but there is not much scientific evidence to verify and validate antidiabetic properties of the plants. Hence, the present study was carried out to determine the potency of *Eugenia jambolana, Centella asiatica, Ocimum sanctum* and *Coccinia indica* extracts and *in vitro* evidences for the anti-diabetic activities.

These plants grow wild in India, but are also widely cultivated. Apart from their significance, these have a long history of medicinal uses. Leaves of *O. sanctum* have been shown to possess hypoglycaemic effects in experimental animals [7, 10, 11 & 12]. *E. jambolana* has been reported to exhibit anti-inflammatory [13], anti-diarrhoeal [14], anti-HIV [15], anti-bacterial [16] and hypoglycemic effects both in experimental and clinical studies [17, 18]. *C. indica* has antidiabetic [19], anti-inflammatory, analgesic, hepatoprotective [20, 21], antioxidant [22], antilithic [23] and antimutagenic [24] activities. In ancient Ayurvedic medicine, *C. asiatica* is reputed to restore youth, longevity [25], antioxidant property [26], memory [27], combating physical and mental exhaustion [28]. Despite these researches, no studies have been performed on carbohydrate enzyme inhibitory activity, insulin secretion and cytotoxicity studies using RIN m5F cells. Hence, these selected plants need to be evaluated scientifically for the anti-diabetic properties.

Materials and Methods

RPMI and DMEM media were procured from Gibco, MTT from Promega, RIN m5Fcells were procured from ATCC, MEM, BSA, Porcine pancreatic α-amylase, α-glucosidase were from Sigma USA, FBS & Antimycotic agents were from Invitrogen, acarbose and 2-chloro-4-nitrophenol α-D-maltotrioside (CNPG3) were obtained from Himedia Laboratory, Mumbai. Insulin ELISA kit were procured from Mercodia (Sweden).

Preparation of solvent extracts

Leaves of *E. jambolana, C. asiatica, O. sanctum* and *C. indica* were collected from Bangalore and other parts of Karnataka, India. Thoroughly washed leaves were shade dried at room temperature and then powdered with the help of a waring blender. Twenty five grams of shade-dried leaf powder was filled in a thimble and extracted with 150 ml of methanol in a soxhlet extraction unit for up to 12 h. The resulting extracts were concentrated separately under reduced pressure and stored in dessicators until further use.

Alpha Amylase assay

Alpha amylase activity was carried out as reported by Roux and co workers [29]. The assay mixture consisting of 120 µl phosphate buffer (40 mM) pH 6.9, along with test sample of various concentrations (dissolved in 1% methanol) and positive control (Acarbose) was pre-incubated with 60 µl of enzyme at 37 °C for 10 minutes. The substrate reagent 250 µl (CNPG3) was added and incubated at 37 ^{o}C for 8 minutes. Control tubes were run devoid of test samples. The absorbance was measured at 405nm using Tecan Microplate Reader.

Alpha Glucosidase assay

Alpha Glucosidase activity was carried out as reported by Matsuo and co workers [30]. The assay mixture consisting of 250 µl phosphate buffer (40 mM) pH 6.9, along with test sample of various concentrations (dissolved in 1% methanol) and positive control (Acarbose) was pre-incubated with 50 µl of enzyme at 37 °C for 30 min. 500 µl of sucrose solution was added and incubated at 37 °C for 20 min, heated on a boiling water bath for 2 min to arrest the reaction and cooled. The glucose concentration was measured by glucose oxidase method.

Glucose oxidase method: 100 µl of the sample was mixed with 500 µl of glucose reagent (Glucose reagent kit) and incubated at room temperature for 10 min. The absorbance was measured at 510nm using Tecan Microplate Reader.

Insulin secretion assay

Insulin secretion assay carried out as reported by Persaud and co-workers [31]. RIN m5F cells (1x 10^5 cells/well) were seeded into the 96 well plate cultured in RPMI 1640 media and incubated for 24 h at 37 ^oC. Before starting the experiment, the cells were washed with KRB buffer, pH 7.4 to remove serum, and incubated for 30 min at 37 ^oC. The different concentrations of test material or positive control (glucose) was prepared in 100 µl KRB buffer and incubated for 4 h at 37 ^oC. Insulin was measured using Mercodia kit. Samples (100 µl) along with peroxidase conjugated anti-insulin antibodies (50 µl) were dispensed into anti-insulin antibodies coated micro titer wells and incubated for 2 h at 37 ^oC. The plate was washed with buffer to remove unbound enzyme labeled antibody. The bound conjugate was reacted with 200 µl of 3,3',5,5'-tetramethylbenzidine (TMB) and incubated for 15 min at room temperature. The reaction was stopped with 50 µl sulphuric acid (0.5 M) and absorbance was measured at 450nm using Tecan Microplate Reader.

Cytotoxicity (MTT) assay

The cytotoxicity effects of the plant extracts on pancreatic β-cells were determined using MTT assay as described by Mossman and co workers [32]. RIN m5F cells (1x10^5 cells/well) were seeded in a 96 well plate. Test material of various concentrations (0, 2.5, 5, 10, 20, 40, 80,160 & 320 µg/mL) were added/well and incubated for 24 h at 37 ^oC for allowing the cells to attach to the bottom of the plate. After incubation, the media was removed from the wells and normal growth media was added. 20 µl/well of the MTT reagent was added and incubated for 3 h. Readings were taken at 485nm using Tecan Microplate Reader.

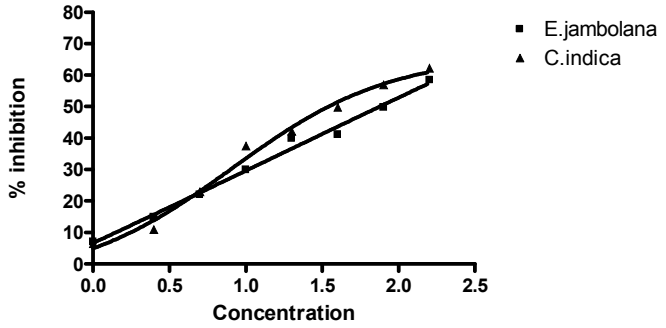
Statistical evaluation

All data are expressed as the mean ± SEM. The Statistical data were evaluated by using Graph pad prism4 software. The IC_{50} value was determined by nonlinear regression curve fit using Graph pad prism4.

Results

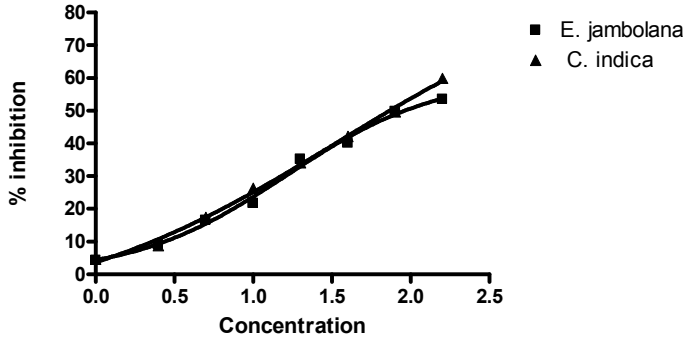
In the present investigation, the inhibitory activities of the methanol extracts of *E. jambolana, C. asiatica, O. sanctum* and *C. indica* were investigated on the α-amylase and α-glucosidase enzymes in comparison to positive control (Acarbose). Among the plants studied, *E. jambolana* and *C. indica* exhibited highest α-amylase and α-glucosidase inhibitory activity. The methanol extract of *C. indica* exhibited highest α-amylase with an IC_{50} of 8.17 µg/mL (Fig. 1) and α-glucosidase inhibitory activity with IC_{50} of 16.75 µg/mL (Fig. 2). The *E. jambolana* extract showed α-glucosidase inhibitory activities with an IC_{50} of 16.27 µg/mL and α-amylase inhibitory activity with IC_{50} of 33.96 µg/mL. In case of *C. asiatica* and *O. sanctum* the extract seems to be less potent in α-amylase and α-glucosidase inhibitory activities. The IC_{50} of acarbose for α-amylase and α-glucosidase inhibition is 2.6 µg/mL and 6.7 µg /mL respectively (Fig. 3).

Fig 1: Alpha amylase inhibition assay



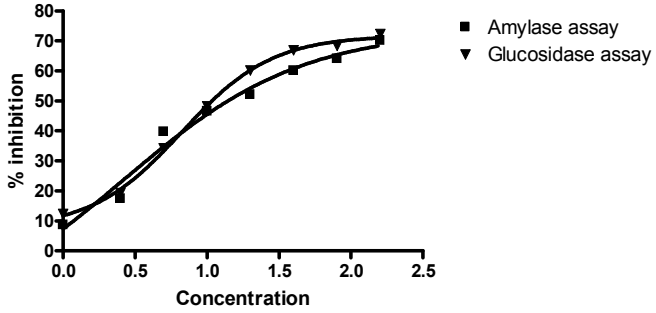
Sigmoidal dose-response (variable slope)	*E. jambolana*	*C .indica*
Best-fit values		
BOTTOM	-12.74	-5.664
TOP	93.23	66.53
LOG EC50	1.531	0.9121
HILL SLOPE	0.4812	0.8382
IC_{50} (µg /mL)	33.96	8.17

Fig.2: Alpha glucosidase inhibition assay



Sigmoidal dose-response (variable slope)	*E. jambolana*	*C. indica*
Best-fit values		
BOTTOM	-0.2813	-112.3
TOP	60.69	182
LOG EC50	1.212	1.224
HILL SLOPE	0.8951	0.1377
IC_{50} (µg /mL)	16.27	16.75

Fig.3: Reference Standard- Acarbose for alpha amylase and alpha glucosidase assay



Sigmoidal dose-response (variable slope)	Amylase assay	Glucosidase assay
Best-fit values		
BOTTOM	-26.74	7.468
TOP	73.84	71.77
LOG EC50	0.4177	0.8257
HILL SLOPE	0.6968	1.387
IC_{50} (µg /mL)	2.617	6.695

Table.1: Insulin release assay in RIN m5f cells using sandwich ELISA method

Sl No.	Test material	Concentration	Fold Stimulation	Insulin Release (µg /L)
1	Control	0	1	
2	Glucose	1 mM	1.98	14.83
		5 mM	2.8	21.09
		10 mM	2.67	20.05
3	*E. jambolana*	50 µg/mL	1.59	11.81
		100 µg/mL	2.23	16.71
4	*C. indica*	50 µg/mL	1.39	10.35
		100 µg/mL	1.12	8.27
5	*C. asiatica*	50 µg/mL	1.80	13.48
		100 µg/mL	0.80	5.76
6	*O. sanctum*	50 µg/mL	1.60	11.91
		100 µg/mL	1.04	7.64

E. jambolana extract showed promising insulin stimulatory effect on RIN m5Fcells in a dose-dependent manner and secreted 2-3 fold at 100 µg/mL when compared to 5mM glucose. The extracts of *C. asiatica, O. sanctum* an*d C. indica*, showed 1-2 fold insulin stimulatory effect at 50 µg/mL. However *C. asiatica* did not show dose dependent stimulation of insulin secretion (Table 1). In order to determine the cytotoxic effects, the extracts were tested on pancreatic β-cells using MTT reagent. All the extracts had no significant adverse effect on viability of pancreatic β-cells.

Discussion

Alpha amylase and alpha glucosidase are responsible for the hydrolysis of poly and oligosaccharides into monomers or cleavage of bonds between sugars and non carbohydrate aglycone. Thus, this enzyme is involved in a number of important biological processes, such as digestion of carbohydrate into glucose [33] or processing of the oligosaccharide moieties of glycoprotein [34]. There is now a great deal of interest in amylase and glucosidase inhibitors, because these are important biochemical tools for studying the mechanism of enzymes. The search for amylase and glucosidase inhibitors has yielded a number of chemically distinct inhibitors from plants [35, 36, 37, 38, 39 & 40].

In the present study, α-amylase was significantly inhibited by *E. jambolana* and *C. indica* in a dose dependent manner. Natural α-amylase inhibitors from food-grade herbal sources offer an attractive therapeutic approach to the treatment of postprandial hyperglycemia by decreasing glucose release from starch and may have potential for use in the treatment of diabetes mellitus and obesity [41]. Results of present investigation agree with other reports [42, 43 & 44].

In order to clarify the postprandial glucose suppression via α-glucosidase inhibitory action by methanol extracts of *E. jambolana* and *C. indica,* showed potent sucrase inhibitory activity, while less inhibitions was observed in *C. asiatica* and *O. sanctum*. The retardation of membrane bound α-glucosidase inhibitory reaction or inhibition of passive glucose transport would successfully flatten the postprandial blood glucose excursions or reduce hyperglycemia. On the basis of the results, *E. jambolana* and *C. indica* had the strongest α-glucosidase inhibitory activity among the four herbal extracts.

Inhibition of α-amylase and α-glucosidase should result in delayed carbohydrate digestion and glucose absorption with attenuation of post prandial hyperglycemic excursions. It has been reported that inhibitors usually do not alter the total amount of carbohydrate absorbed and therefore do not cause any net nutritional caloric loss although they slow down carbohydrate digestion. Postprandial hyperglycemia could induce the non-enzymatic glycosylation of various proteins, resulting in the development of chronic complications. Therefore, control of postprandial plasma glucose levels is critical in the early treatment of diabetes mellitus and in reducing chronic vascular complications.

An antidiabetic agent could exert a beneficial effect in the diabetic situation by enhancing insulin secretion and/or by improving/mimicking insulin action [41]. Sulphonylurea-induced insulin secretion by clamping open ATP-sensitive K^+ channels, thus preventing membrane depolarization and subsequent Ca^{2+} influx, two of the key initial steps in insulin secretion [45]. In the present study, *E. jambolana* has significant effects on insulin secretion and stimulated more than 2-fold insulin release. The extract is therefore likely to act at an early stage of the insulin secretory pathway before Ca^{2+} influx. These characteristics are reminiscent of sulphonylureas, these studies show that the anti-hyperglycemic actions of *E. jambolana* are associated with the stimulation of insulin secretion.

Assessing cell membrane integrity is one of the most common ways to measure cell viability and cytotoxic effects. Compounds that have cytotoxic effects often compromise cell membrane integrity. In the present experiment, all methanol extracts were tested up to 320 µg/ml in the MTT assay for cell viability. The cell viability was 100% in all the concentrations, further evidence suggests that the extracts are less toxic and enhanced the secretion of insulin.

Conclusion

This study is significant as it covers various important biochemical and metabolic aspects responsible for the progression of diabetes. These plant extracts have definitely shown multiple biological targets however, at this stage it is difficult to predict whether all the components act independently or in a synergetic manner because active principles or biomolecules are responsible for their antidiabetogenic effect, which is required to be identified. Therefore methanol extracts of *C. indica* and *E. jambolana* are selected for further investigation, involving bioassay guided fractionation, in order to isolate and characterize the active compound in search of novel antidiabetic agents that holds the hope of new generation antidiabetic drugs.

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